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Characterization of a Positive Regulatory Gene, *LAC9*, That Controls Induction of the Lactose-Galactose Regulon of *Kluyveromyces lactis*: Structural and Functional Relationships to *GAL4* of *Saccharomyces cerevisiae*

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Lactose or galactose induces the expression of the lactose-galactose regulon in *Kluyveromyces lactis*. We show here that the regulon is not induced in strains defective in *LAC9*. We demonstrate that this gene codes for a regulatory protein that acts in a positive manner to induce transcription. The *LAC9* gene was isolated by complementation of a *lac9* defective strain. DNA sequence analysis of the gene gave a deduced protein of 865 amino acids. Comparison of this sequence with that of the GAL4 protein of *Saccharomyces cerevisiae* revealed three regions of homology. One region of about 90 amino acid occurs at the amino terminus, which is known to mediate binding of GAL4 protein to upstream activator sequences. We speculate that a portion of this region, adjacent to the "metal-binding finger," specifies DNA binding. We discuss possible functions of the two other regions of homology. The functional implications of these structural similarities were examined. When *LAC9* was introduced into a *gal4* defective strain of *S. cerevisiae* it complemented the mutation and activated the galactose-melibiose regulon. However, *LAC9* did not simply mimic *GAL4*. Unlike normal *S. cerevisiae* carrying *GAL4*, the strain carrying *LAC9* gave constitutive expression of *GAL1* and *MEL1*, two genes in the regulon. The strain did show glucose repression of the regulon, but repression was less severe with *LAC9* than with *GAL4*. We discuss the implications of these results and how they may facilitate our understanding of the LAC9 and GAL4 regulatory proteins.

Elucidation of genetic regulatory systems has relied heavily on the isolation of mutations in regulatory and structural genes. We are utilizing this approach to determine how lactose or galactose induces the lactose-galactose regulon in the yeast *Kluyveromyces lactis*. The regulon contains five known structural genes whose products are: *LAC4*, β-galactosidase (EC 3.2.1.23) (44); *GAL1*, galactokinase (EC 2.7.1.6) (37); *GAL7*, galactose-1-phosphate uridyltransferase (EC 2.7.7.10) (37); *GAL10*, uridine diphosphoglucose-4-epimerase (EC 5.1.3.2) (37); and *LAC12*, a lactose permease (45). β-Galactosidase activity can be induced over 100-fold above a moderate basal level (12). Increased enzyme activity results from increased transcription of the β-galactosidase structural gene, indicating that induction is regulated at the level of transcription (26). Mutations in *LAC10* cause constitutive expression of the regulon, suggesting that the gene functions in a negative fashion to regulate transcription (14).

To further understand how lactose induces gene expression we characterized in more detail our previously isolated Lac$^-$ mutants (43). We found that mutants defective in *lac9* are uninducible for all the enzymes of the lactose-galactose regulon. These and other data indicate that *LAC9* is a positive regulatory gene that acts in *trans* to control transcription of target genes. This gene is the first positive-acting regulatory function that has been identified for the lactose-galactose regulon of *K. lactis*.

We previously noted both organizational and phenomenological similarities between the lactose-galactose regulon of *K. lactis* and the melibiose-galactose regulon of *Saccharomyces cerevisiae* (37). In the present work we examined the similarities further by comparing the structure and function of *LAC9* and *GAL4*. *GAL4* is a *trans*-acting positive regulatory gene that controls transcription of the melibiose-galactose regulon of *S. cerevisiae* (15, 20). The GAL4 protein binds to one or more 17-base-pair DNA sequences, the upstream activator sequences (UASs), located in front of each structural gene in the regulon (4, 5, 17). It is not known how GAL4 activates transcription, but binding to UASs is not sufficient for gene activation (6). It appears that the UAS-bound GAL4 protein contacts other proteins to activate transcription (24). The DNA-binding domain of GAL4 protein has been localized to a region within the 74 amino-terminal amino acid residues (24). It has been noted that this region contains an amino acid sequence of the form Cys-X_{2-4}-Cys-X_{2-15}-Cys-X_{2-4}-Cys which is found in two other yeast positive regulatory proteins, *ADR1* and *PPR1* (18). This sequence is related to a sequence found in many eucaryotic proteins that bind nucleic acids (3). It has been hypothesized that the four Cys (or His) residues complex with Zn^{2+} and produce a looped-out region of amino acids that contacts nucleic acids; consequently such regions have been termed metal-binding fingers or domains (35).

Other aspects of the structure and function of *GAL4* have been examined. The transcriptional activating function of GAL4 protein is only operational when cells are grown in the presence of an inducer such as galactose. This function of GAL4 protein is thought to be modulated by direct interaction with the GAL80 protein (21), a negative-acting regulatory protein (16, 47).

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TABLE 1. Strains of *K. lactis* used in these studies

Strain	Genotype	Source
Y1140	**a** *lac1 LAC2*	19
5C170	*his2-2 trp2-1 ura1-1*	This study
6C170	*ura1-1*	This study
3B212	*ade3-1 met2-2*	This study
10A212	*ade3-1 met2-2*	This study
MS425	α *lac4-8 ade1-1*	44
2B298	*lac4-8 trp2-1*	This study
AS1D	α *lac5^c-10 gal7-10 ade1-1*	44
MS12	*a gal10-1 met1-1*	44
MS25	**a** *lac9-2 his2-2*	44
MS26	α *lac9-3 ade1-1*	44
3D261	*lac9-2 trp2-1*	This study
15B261	*lac9-2 his2-2 ura1-1*	This study
18D261	*lac9-2 ade1-1 his2-2*	This study
2C275	*lac9-2 met2-2 trp2-1*	This study
MS5060	*lac10-1 ade1-1*	14
11A173	*lac10-1 ade1-1 met2-2*	This study
14B173	*lac10-1 met2-2 trp2-1*	This study
2A276	*lac10-1 ade1-1 his2-2*	This study
6D176	*lac10-1 met2-2 trp2-1*	This study
5C282	*lac12-230 ade1-1 ura1-1*	This study
5D282	*lac12-230 his2-2*	This study
3B303	*lac12-230 ade3-1 ura1-1*	This study
11C303	*lac12-230 ade3-1 met2-2*	This study
11D304	*lac12-230 his2-2*	This study
16C492	*lac12-101 trp2-1*	This study
16D492	*lac12-101 his2-2 trp2-1*	This study
6B191	*gal7^c-10 lac9-2 ade1-1 his2-2*	This study
6C191	*gal7^c-10 lac9-2 ade1-1*	This study
14B282	*gal7^c-10 lac12-230 trp2-1 ura1-1*	This study
14D282	*gal7-10 lac12-230 trp2-1 ura1-1*	This study
1C305	*lac9-2 lac12-230 ade1-1 ura1-1*	This study
1D305	*lac9-2 lac12-230 ade1-1 trp2-1*	This study
3B306	*lac9-2 lac12-230 trp2-1*	This study
3C306	*lac9-2 lac12-230 his2-2*	This study
9B383	*lac9-3 ura3-1 met2-2*	This study
10B383	*ura3-1 met2-2*	This study
7B520	*ura3-1 his2-2 trp1*[a]	This study

[a] This *trp1* allele is described by Das and Hollenberg (10).

Comparison of the predicted LAC9 and GAL4 protein sequence revealed that the first 94 amino acids in the GAL4 protein are highly homologous to residues 85 through 178 in the LAC9 protein. This region contains both the metal-binding finger and an adjacent region of unknown function. Two other portions of the proteins are homologous including a region of about 160 residues in the middle of the protein and a 16-residue region at the carboxy terminus.

The functional implications of these structural similarities were assessed by measuring the ability of *LAC9* to complement a *gal4* mutant strain of *S. cerevisiae*. *LAC9* complemented *gal4* and activated expression of the melibiose-galactose regulon. However, it did not simply mimic *GAL4*. *LAC9* gave constitutive expression of the regulon in contrast to the nonconstitutive and highly inducible expression mediated by *GAL4*. The regulon was repressed by glucose in the presence of either gene, but repression was greater with *GAL4*.

The structural and functional similarities and differences between *LAC9* and *GAL4* should be useful in determining how proteins with metal-binding fingers recognize specific DNA sequences, how these regulatory proteins connect to other components of their respective regulons, and how the regulons interface to global regulatory circuits including carbon catabolite repression.

MATERIALS AND METHODS

Strains and media. The *K. lactis* strains used in these studies are listed in Table 1. The *K. lactis trp1* mutation described by Sheetz and Dickson (43) was redesignated here as *trp2* so that it is consistent with *S. cerevisiae* gene assignments. *S. cerevisiae* SJ21 (*gal4 ura3 leu2 ade1*) was provided by J. Hopper (21). The compositions of YMPD complex medium and minimal media have been described previously (37).

Intracellular galactose. Strain MS25 was grown at 30°C to saturation in double-strength yeast nitrogen base (Difco Laboratories, Detroit, Mich.) containing 20 mM sorbitol, 100 mM galactose, and 40 μg each of adenine, uracil, His, Lys, Met, and Trp per ml. Cells were diluted with fresh medium and grown from 0.5 to 1.0 A_{600} units per ml. They were filtered and suspended in buffered medium at 1.0 A_{600} unit per ml as described previously (37). D-[^{14}C]galactose (CMM-264; 45 mCi/mmol; Research Products International, Mount Prospect, Ill.) was added to a final concentration of 0.83 μCi/ml, and the 15-ml culture was grown for 6 h. The

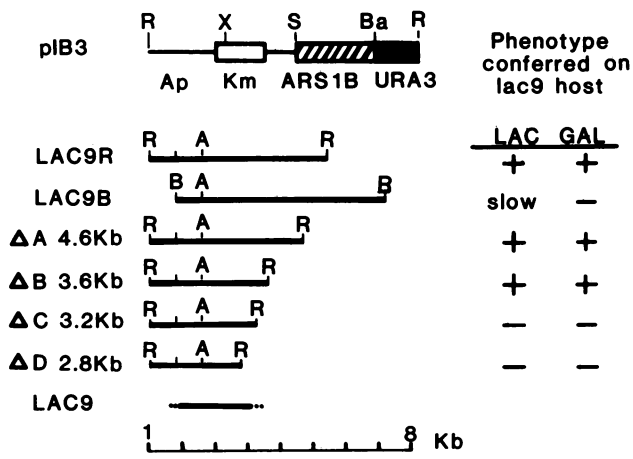


FIG. 1. Subcloning of *LAC9*. The location of *LAC9* on the 13-kb yeast DNA insert in pIB3-M2 (not shown) was determined by first cloning portions of the insert into the *Eco*RI or *Bgl*II site of pIB3 and then examining the ability of the subclones to complement the *lac9* mutation in strain 9B383. Complementation was scored by measuring growth of cells transformed with pIB3 carrying the indicated DNA fragments on MinLac or MinGal plates. The subclones ΔA, ΔB, ΔC, and ΔD were obtained by the procedure of Dale et al. (8) to make deletions of the *LAC9R* fragment carried in M13mp19. The most probable location of *LAC9* is shown at the bottom of the figure. Abbreviations for restriction endonucleases are: A, *Asp* 718; B, *Bgl*II; Ba, *Bam*HI; R, *Eco*RI; S, *Sal*I; X, *Xho*I. Abbreviations for pIB3 are: solid thin line, pBR322 sequences; Ap, ampicillin resistance marker; open box, Tn*903* sequences which yield kanamycin (Km) resistance in *E. coli* and G418 resistance in yeasts; cross-hatched box, *K. lactis ARS1B*; solid box, *S. cerevisiae URA3* bounded by *Hin*dIII sites and inserted into the *Hin*dIII site of pBR322. The unique *Bgl*II site in pIB3 is located within *ARS1B* about 1 kb from the *Bam*HI site.

TABLE 2. Specific enzyme activities in mutant strains

lac genotype	No. of determinations	Sp act[a]					
		β-Galactosidase	Galactokinase	Transferase	Eipmerase	Phosphoglucomutase	Alkaline phosphatase
Wild type (Y1140)	7	2,340	46	50	6.0	97	51
		407	9.8	6.4	1.2	91	45
lac9-2 (MS25)	2	76	1.6	1.4	0.3	101	57
		95	1.1	0.8	0.2	114	34
lac9-3 (MS26)	2	33	0.5	1.1	0.1[b]	82	59
		89	0.9	1.4	0.1[b]	92	31

[a] Specific activities (nanomoles per minute per milligram of protein) were determined on cell extracts prepared from log-phase cells grown at 30°C in minimal medium containing 20 mM glucose. Values above the line are for cells induced by the addition of 40 mM galactose, while values below the line are for uninduced cells. Typically the values had a 20 to 30% standard deviation.

[b] These values are below the detection limit of 0.2 nmol/min per mg.

cells were then filtered, washed, and ethanol extracted, and the ethanol extract was chromatographed on Whatman 3MM paper as previously described (11). The spot containing lactose was identified by comparing its mobility with that of a lactose standard. The spot was cut out and counted in a liquid scintillation counter.

Construction of *K. lactis* recombinant DNA library. High-molecular-weight DNA from wild-type *K. lactis* Y1140 was isolated and purified on a cesium chloride gradient by the method of Kaback and Davidson (22). This DNA was partially cut with the restriction endonuclease *Sau*3A (1 unit/25 μg for 3 to 5 min) and size fractionated on a 10 to 40% sucrose gradient. Fractions estimated to be between 12 and 20 kilobases (kb) by gel electrophoresis were pooled and ligated into the *Bam*HI site of p1B3. Self-ligation of the vector was prevented by treatment with alkaline phosphatase. The vector p1B3 (Fig. 1) is a pBR322 derivative. It contains two yeast-selectible markers: *URA3* (39) for selection of Ura$^+$ transformants in a *ura3* host, and TN*903* for selection of G418-resistant transformants (46). This ligation mix was used to transform DG75 (13) to ampicillin resistance (Amp^r). Approximately 30,000 Amp^r transformants were pooled to make the library. About 90% of the Amp^r transformants contained an insert.

DNA sequencing. Various DNA fragments containing portions of the *LAC9* region were cloned into M13mp18 or M13mp19 (50). Deletions of these clones were generated by the method of Dale et al. (8). Single-stranded bacteriophage templates were sequenced by the dideoxy chain termination method (41) with either avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) or *Escherichia coli* DNA polymerase I Klenow fragment (Pharmacia, Inc., Piscataway, N.J.). Both strands of the sequence reported here were independently determined.

Construction of the *LAC9*::*URA3* disruption. The 5.4-kb *Eco*RI *LAC9* fragment from p1B3-*LAC9*R was cloned into pIC20R (31) to give pRS2. The only *Asp* 718 restriction site within this plasmid is located in the *LAC9* gene. The plasmid pUC-*URA3* is a derivative of pUC18 and pUC19 (50) containing the *S. cerevisiae URA3* gene on a 1.1-kb *Hin*dIII fragment located between symmetrical restriction site polylinkers in which the *Hin*dIII site is in the center of the linker and the *Eco*RI sites are at the outer edges. A 1.1-kb *Asp* 718 *URA3* DNA fragment from this plasmid was inserted into the *Asp* 718 site of pRS2 to give pL9AU1. The wild-type *LAC9* gene was transplaced (40) by transforming *K. lactis* 7B520 with *Eco*RI-digested pL9AU1 and selecting for Ura$^+$ colonies. The structure of several integrants was confirmed by Southern blot analysis.

Construction of YCp50 derivatives. The *S. cerevisiae* cloning vector YCp50 is a single-copy plasmid that contains *ARS1*, *CEN4*, and *URA3*. This plasmid can also replicate in *E. coli* and confer resistance to ampicillin and tetracycline. YCp50-*GAL4* contains the *GAL4* gene from pSJ4 (21) on a 3.5-kb *Bam*HI-*Sau*3AI fragment inserted into the *Bam*HI site of YCp50. Both of these plasmids were obtained from James Hopper. To insert the *LAC9* gene into YCp50 at the same position and with the same orientation as *GAL4*, we first cloned the 5.4-kb *LAC9 Eco*RI fragment (Fig. 1) into the plasmid pUC19-18. This plasmid is a derivative of pUC18 and pUC19 (50) in which the restriction site polylinkers are symmetrically located with the *Eco*RI site in the center and the *Hin*dIII sites at the outer edges. A *Bam*HI fragment containing the *LAC9* gene was then inserted into the *Bam*HI site of YCp50. The orientation of the *LAC9* DNA fragment was determined by restriction mapping.

Miscellaneous procedures. Procedures for preparing cell extracts for enzyme assays, for genetic crosses, and for complementation tests have been described previously (37). *K. lactis* was transformed to Ura$^+$ by the method of Sreekrishna et al. (46) except that 60% instead of 40% polyethylene glycol 4000 (BDH, Poole, England) was used. Total RNA was isolated by the method of Carlson and Botstein (7).

RESULTS

Characterization of mutants defective in *LAC9*. Mutants defective in *LAC9* were originally identified as Lac$^-$ isolates of *K. lactis* Y1140 (43). To further characterize *lac9* defective strains we measured their uninduced and induced levels of lactose (galactose)-inducible enzymes. As the data in Table 2 indicate, in *lac9* defective strains there was no induction of any of the enzymes measured. Induction actually caused a slight reduction in the activity of some enzymes compared with the uninduced level. These results suggest that *LAC9* regulates induction. The uninduced level of enzymes was lower in *lac9* defective strains than in the wild type. This result implies that *LAC9* protein plays a role in setting the uninduced as well as the induced level of gene expression. In these experiments phosphoglucomutase served as a control for an uninducible enzyme related to the galactose catabolic pathway, and alkaline phosphatase served as a control for an uninducible enzyme unrelated to

TABLE 3. Genetic mapping of *LAC9*

Cross[a]	Phenotype[b]	Type of tetrad			Map distance in centimorgans
		Parental ditype	Tetratype	Nonparental ditype	
lac9- × *lac4-*	1	10	16	6	No linkage
lac9- × *gal7-*	1	15	56	12	No linkage
lac9- × *gal10-*	1	2	0	0	No linkage
lac9- × *lac12-*	1	6	18	9	No linkage
lac10-1 × *gal7-*	2	6	20	6	No linkage
lac10-1 × *lac12-230*	3	6	24	8	No linkage
lac9-2 × *lac10-1*	3	50	2	2	
lac9-3 × *lac10-1*	3	28	2	0	
lac9-12 × *lac10-1*	3	10	2	3	
lac9-9 × *lac10-1*	3	48	0	0	
		136	6	5	12.2

[a] When no allele is given, several different alleles were used.

[b] Phenotypes used to assign the type of tetrad are: 1, parental ditype (PD) ($0Lac^+:4Lac^-$), tetratype (T) ($1Lac^+:3Lac^-$), nonparental ditype (NPD) ($2Lac^+:2Lac^-$); 2, PD ($0Lac^+:2Lac^{-c}:2Lac^c$), T ($1Lac^+:2Lac^{-c}:1Lac^c$), NPD ($2Lac^+:2Lac^{-c}:0Lac^c$); 3, PD ($0Lac^+:2Lac^-:2Lac^c$), T ($1Lac^+:2Lac^-:1Lac^c$); NPD ($2Lac^+:2Lac^-:0Lac^c$). The constitutive phenotype, Lac^c, is defined as higher β-galactosidase activity in the absence of inducer in a 5-bromo-4-chloro-3-indolyl-β-D-galactoside plate assay (14) compared with that of the Lac^+ wild type.

lactose or galactose metabolism. Neither enzyme activity was inducible or affected by a mutation in *lac9*.

An alternative explanation for the function of *LAC9* is that it facilitates transport of lactose and galactose, so that in a *lac9* defective strain neither sugar is transported. Consequently, the lactose-inducible enzymes are not induced. This seems unlikely since galactose transport is detectable in *lac9* defective cells (11) and *LAC12* has been identified as the structural gene for lactose permease (45). Nevertheless, we ruled out this possibility by measuring the intracellular concentration of D-[^{14}C]galactose (37). After a 6-h uptake the intracellular concentration of ethanol-extractable galactose was about 150 mM. We believe that this concentration is sufficiently high to induce fully all the enzymes shown in Table 2, since full induction of the enzymes occurs in wild-type cells in which the intracellular pool of galactose is less than 5 mM (M. I. Riley and R. C. Dickson, unpublished data). Since there is measurable activity of all lactose-inducible enzymes in *lac9* defective strains but the activities are uninducible, and since the intracellular level of galactose is sufficient to induce the lactose-galactose regulon but does not, we propose that *LAC9* is a regulatory, rather than a structural, gene.

Mapping of *LAC9* by tetrad analysis. Genetic mapping by tetrad analysis showed that *LAC9* was unlinked to *LAC4*, *LAC12*, or the galactose gene cluster containing *GAL1*, *GAL7*, and *GAL10* (Table 3). Close linkage, 12.2 centimorgans, to *LAC10* was found (Table 3). However, the linkage may be closer owing to loss of the *lac10* marker in these crosses. For example, some auxotrophic markers were absent in 5 to 10% of the tetrads examined. A loss of marker genes could be due to mitotic crossing-over before meiosis. If *lac10* was lost at a similar rate, the frequency of the nonparental ditype class would increase, which is what we observed (Table 3). If we take this into account, the linkage between *LAC9* and *LAC10* is about 2 centimorgans. Also, we determined that the two *lac9* alleles used here were recessive to *LAC9*. The phenotypes of spores from a tetratype ascus suggest that *lac9* (the Lac^- phenotype) is epistatic to *lac10* (the Lac^c phenotype). Further experiments will be needed to verify this possibility.

Isolation of *LAC9*. *LAC9* was isolated by complementation of a *lac9* mutant strain. For this experiment strain 9B383 *lac9 ura3* was transformed with a *K. lactis* recombinant DNA library, and the 20,000 Ura^+ transformants obtained were pooled. Lac^+ colonies were selected from the pool on minimal lactose (MinLac) plates. Total DNA was prepared from several Lac^+ colonies which showed instability for the Ura^+ and Lac^+ phenotypes. Instability indicated that the phenotypes were mediated by a plasmid. The DNA was used to transform *E. coli* to ampicillin resistance. Several unique bacterial clones were isolated that gave plasmid DNA which complemented the *lac9* and *ura3* defects after retransformation into *K. lactis* 9B383. One plasmid, p1B3-M2 (not shown), contained a 13-kb insert. To ensure that p1B3-M2 contained *LAC9* and not some other regulatory gene, we examined its ability to integrate at the chromosomal *LAC9* locus (36). This was accomplished by growing three independent transformants of 9B383 harboring p1B3-M2 nonselectively for 30 generations in YPD medium, screening stable Ura^+ transformants, and genetically mapping the Lac^+ phenotype by crossing strains to a *ura3 LAC9* strain (7B520). Integrated p1B3-M2 behaved as a single locus since all 23 tetrads segregated $2Ura^+:2Ura^-$ spores. All spores were Lac^+, indicating a parental ditype configuration and tight linkage of the integrated p1B3-M2 to *LAC9*.

Delineation of *LAC9*. Subclones derived from p1B3-M2 were used to localize the *LAC9* gene (Fig. 1). A 5.4-kb *Eco*RI fragment was inserted into p1B3 at the unique *Eco*RI site to give p1B3-*LAC9R*. This subclone gave good complementation of *lac9* as determined by growth on MinLac and minimal galactose (MinGal) plates. Another subclone, p1B3-*LAC9B*, containing a 6.3-kb *Bgl*II fragment, gave only weak complementation of *lac9* on MinLac plates and no complementation on MinGal plates. This phenotype suggests that one of the *Bgl*II sites lies close to or within the *LAC9* gene. This is presumably the *Bgl*II site located within the 5.4-kb *LAC9R* subclone that complements *lac9* (Fig. 1).

Deletions extending inward from the right side of the 5.4-kb *Eco*RI fragment were generated in vitro (8), inserted into the *Eco*RI site of p1B3, and used to localize further the *LAC9* gene. The ΔA and ΔB deletions (Fig. 1), which contained 4.6 and 3.6 kb of DNA, respectively, complemented *lac9* for growth on MinLac and MinGal plates. Deletions containing either 3.2 or 2.8 kb of DNA (ΔC and ΔD; Fig. 1) did not complement *lac9*. Taken together, these

data suggest that the *LAC9* gene is located in the region shown in Fig. 1.

Disruption of chromosomal *LAC9* gene. To further verify that *LAC9* had been isolated, we used the cloned gene to disrupt its chromosomal homology (40). A 1.1-kb *Asp* 718 fragment encoding the *URA3* gene of *S. cerevisiae* was inserted into the *Asp* 718 site of the 5.4-kb *Eco*RI fragment carrying *LAC9*. The resulting DNA fragment was used to replace the wild-type gene of strain 7B520 by selecting for uracil prototrophy. Since the *Asp* 718 site should lie within the *LAC9* gene, integration of the *URA3*-disrupted gene at the homologous chromosomal location should give a *lac9* phenotype. Southern blot analysis of two independent integrants with drastically reduced ability to utilize lactose and galactose confirmed that the *URA3* gene had been inserted into the chromosomal 5.4-kb *LAC9 Eco*RI fragment (data not shown). The phenotype of the two *lac9::URA3* disruption strains, L9AU7 and L9AU12, was examined by measuring β-galactosidase and galactokinase activity. Neither enzyme was induced by galactose (Table 4). Thus the two *URA3* disruption strains behaved like previous *lac9* alleles (Table 2). Finally, we showed that p1B3-*LAC9R* complemented strains L9AU7, L9AU12, and 9B383 (*lac9-3*) and allowed growth on MinLac plates. Complementation argues that the *lac* mutations in all three strains occur in the same complementation group or gene since, as we show below, the 5.4-kb *LAC9R* DNA fragment only codes for one detectable RNA, which codes for the LAC9 protein.

Characterization of *LAC9* transcription. The direction of *LAC9* transcription was determined by using probes specific for each strand of DNA. The *LAC9R* DNA fragment was cloned into M13mp19 in both orientations to yield the strand-specific probes which were used for Northern blot analysis (Fig. 2, LAC9R-T and LAC9R-B). Only the LAC9R-B probe hybridized; it hybridized to an RNA of 2.9 kb (Fig. 2, lanes 1 and 2). Thus the direction of *LAC9* transcription is from left to right in Fig. 2. In addition, the 2.9-kb band appears to be two- to threefold inducible as determined from densitometry scans of the autoradiograms shown in Fig. 2.

Transcription start sites were determined by a primer extension procedure (34). The primer 5′-GGCAGTAACG TTTCCGCC-3′ was labeled at its 5′ end with [γ-^{32}P]ATP and T4 bacteriophage polynucleotide kinase. The labeled primer was annealed to poly(A)$^+$ RNA isolated from *K. lactis* Y1140 (26) and extended with avian myeloblastosis virus reverse transcriptase. The nucleotide at which primer extension reactions terminated was determined by comparison with a set of dideoxy DNA-sequencing reactions in which nonradioactive primer and an M13mp19-*LAC9* DNA single-stranded template was used. The results of these experiments are shown in Fig. 2, and the transcription start sites are indicated by asterisks on the *LAC9* DNA sequence shown in Fig. 3.

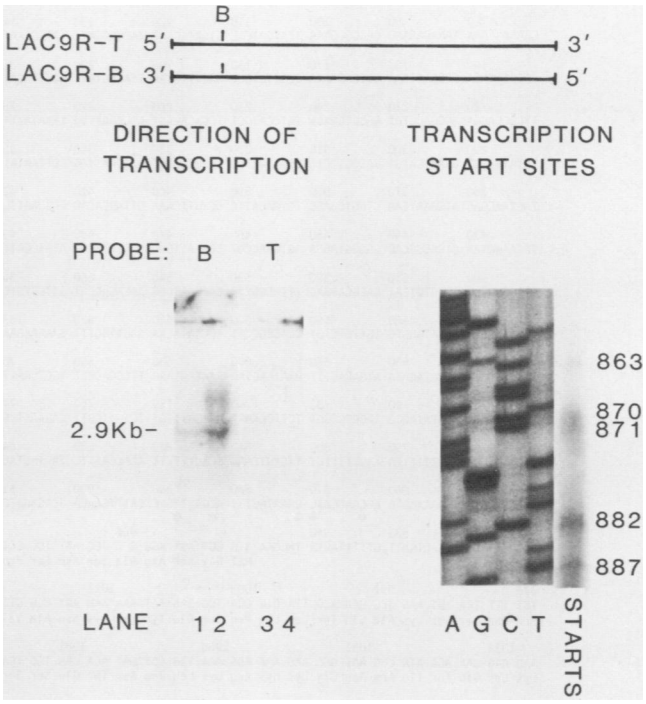


FIG. 2. Analysis of *LAC9* transcription. The size of the *LAC9* message and its direction of transcription were determined with Northern blots. Total RNA was isolated from *K. lactis* 10B383 grown on minimal medium containing 2% sorbitol under uninduced conditions (odd-numbered lanes) or induced conditions (2% galactose; even-numbered lanes). The RNA was separated on 1.2% agarose gels containing 6% formaldehyde (30) and prepared for Northern blot analysis with nitrocellulose paper (30). The single-stranded probes LAC9R-T and LAC9R-B, carried in opposite orientations in M13mp19, were first hybridized to a Northern blot, and then the blot was rehybridized to denatured double-stranded ^{32}P-labeled M13mp19 DNA. Molecular weights were determined with the 18S and 25S rRNAs, the 2.9-kb *GAL4* mRNA (27), and the 3.0-kb *LAC4* RNA (10; R. C. Dickson, unpublished data) as molecular weight standards. Transcription start sites were determined by a primer extension procedure (34). An autoradiogram of a DNA-sequencing gel on the right side of the figure shows the products of the primer extension reaction (STARTS) and the products of the DNA-sequencing reactions (A, G, T, C). The numbers to the right of the autoradiogram refer to the *LAC9* DNA sequence shown in Fig. 3.

TABLE 4. Disruption of *LAC9* with *URA3* prevents enzyme induction

Strain	*lac* genotype	Sp act[a]	
		β-Galactosidase	Galactokinase
7B520	*LAC9*	3,495	8.1
		128	3.7
9B383	*lac9*	16	0.6
		48	2.6
L9AU7	*lac9::URA3*	8.8	0.4
		64	2.8
L9AU12	*lac9::URA3*	17	0.5
		43	3.6

[a] Specific enzyme activities (nanomoles per minute per milligram of protein) were determined on cell extracts prepared from log-phase cells grown on minimal sorbitol medium (2% sorbitol) supplemented with amino acids at 30°C. Values above the line are for cells induced by the addition of 2% galactose, while values below the line are for uninduced cells.

DNA sequence of *LAC9*. The nucleotide sequence of the *LAC9* gene is shown is Fig. 3. The only large open reading frame present within the 3.7-kb DNA sequence starts with the ATG codon at position 947 and ends at the TAA termination codon at position 3542. The size of this 865-codon-long open reading frame is consistent with the size of the 2.9-kb *LAC9* mRNA. A sequence homologous to the UAS of the *K. lactis* lactose-galactose regulon and the *S. cerevisiae* melibiose-galactose regulon is located upstream of the *LAC9* gene at position 533. This sequence may be responsible for the observed induction of the *LAC9* mRNA.

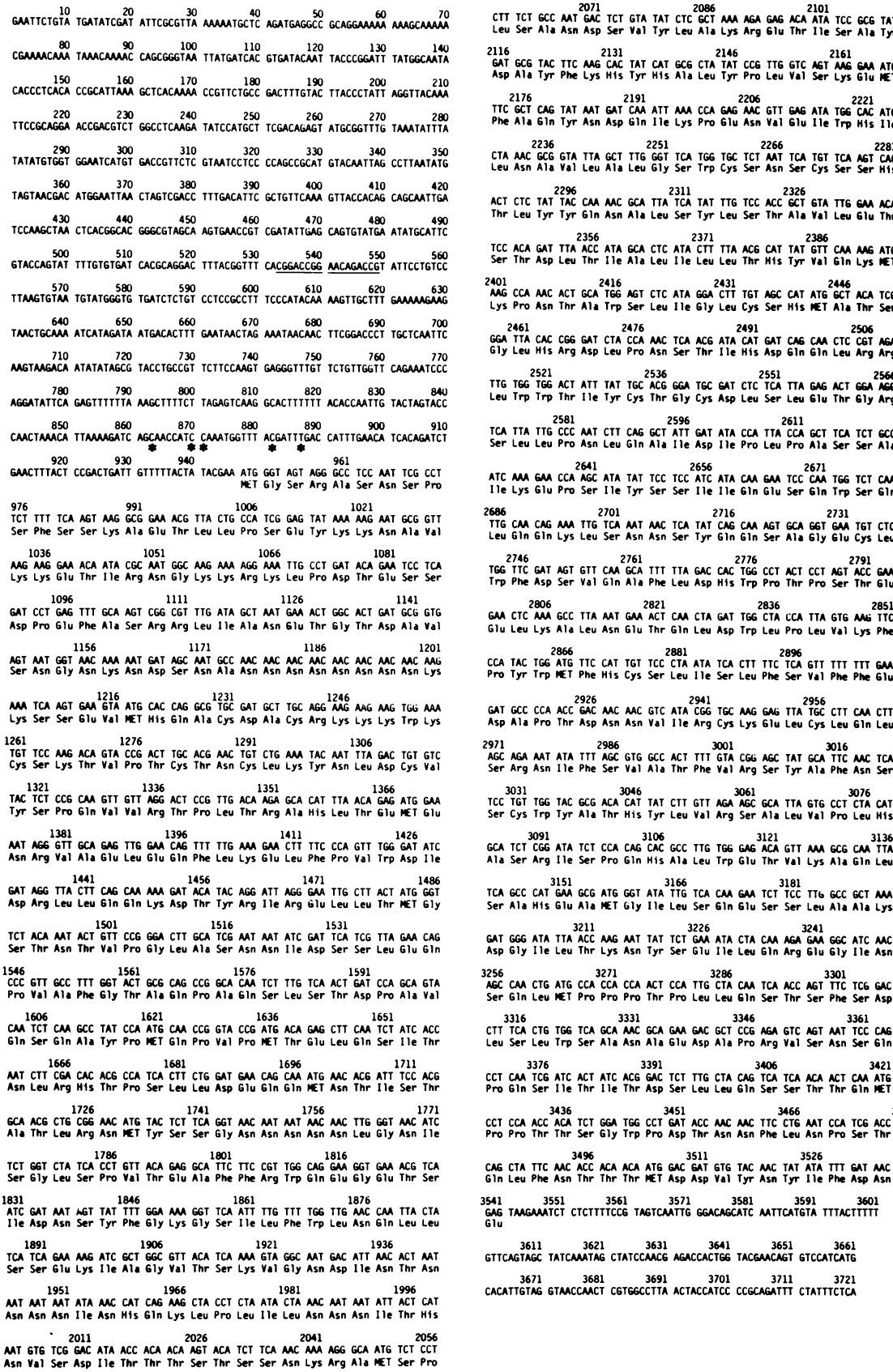


FIG. 3. *LAC9* DNA sequence. The nucleotide sequence of the *LAC9* gene of *K. lactis* is presented along with the protein sequence of the 865-codon open reading frame. The underlined sequence from positions 533 to 549 is homologous to the UAS in the galactose-melibiose regulon of *S. cerevisiae* (5, 17). Nucleotides 1 to 6 represent the *Eco*RI site at the left end of the *LAC9R* DNA fragment shown in Fig. 1. The asterisks below a nucleotide indicate the transcription start points determined as described in the legend to Fig. 2.

TABLE 5. Effect of *LAC9* in a *gal4* strain of *S. cerevisiae*

Growth conditions	Sp act[a]					
	α-Galactosidase			Galactokinase		
	YCp50-*LAC9*	YCp50-*GAL4*	YCp50	YCp50-*LAC9*	YCp50-*GAL4*	YCp50
Uninduced	769	13	4.8	966	<0.5	<0.5
Induced	987	912	4.9	600	700	<0.5
Repressed (glucose)	15.5	4.5	0.8	76	<0.5	<0.5
Repressed (glucose + galactose)	19.9	4.4	0.8	38	<0.5	<0.5

[a] Specific enzyme activities (nanomoles per minute per milligram of protein) were determined on cell extracts prepared from log-phase SJ21 (*gal4*) cells transformed with the indicated plasmid. Cells were pregrown to saturation in supplemented minimal medium containing 2% glucose but lacking uracil to select for the *URA3* gene on YCp50. Saturated cultures were then diluted into media with the following carbon sources: uninduced, 3% glycerol plus 2% lactic acid; induced, 3% glycerol plus 2% lactic acid plus 2% galactose; repressed (glucose), 2% glucose; repressed (glucose + galactose), 2% glucose plus 2% galactose. The initial cell density of the diluted cultures was 0.1 to 0.2 A_{600} units for uninduced and 0.02 to 0.07 A_{600} units for all others. Cell extracts were prepared when the A_{600} reached 0.8 to 1.1, a period of 18 to 21 h. Values represent the average from two experiments in which the variation from the average was less than ±20%.

An *S. cerevisiae* transcription termination-polyadenylation sequence, TAGT. . .TTT (51), is located downstream from the *LAC9* gene starting at position 3562.

Complementation of *S. cerevisiae gal4* gene by *LAC9*. As discussed in the introduction (also see reference 37), there were several reasons for thinking that *LAC9* and *GAL4* share some functions. To determine whether *LAC9* could complement *gal4*, we introduced *LAC9* into a *gal4* deficient strain of *S. cerevisiae* on a vector that is maintained as a single copy per cell, YCp50-*LAC9*. Transformants of SJ21 were selected first for complementation by a vector borne marker, *URA3*, and then purified transformants were tested for complementation by growth on MinGal plates. Under these conditions YCp50-*LAC9* transformants grew as rapidly as YCp50-*GAL4* transformants, and each gave 1- to 2-mm-diameter colonies after 20 h of incubation at 30°C. Controls transformed with YCp50 did not grow on MinGal plates. Thus *LAC9* complements *gal4* for growth on galactose and is able to activate transcription of the galactose regulon of *S. cerevisiae*.

To quantitate the level of transcriptional activation of *GAL1* and *MEL1* (25) by *LAC9*, we measured galactokinase and α-galactosidase activity, respectively, under uninduced and induced conditions. The results presented in Table 5 demonstrate several important points. First, in *LAC9* transformants, galactokinase and α-galactosidase activity was high even in the uninduced state; thus, *LAC9* activated transcription of *GAL1* and *MEL1* constitutively. Second, the induced enzyme levels produced by *LAC9* were about the same as those produced by *GAL4*. Finally, glucose repression of the enzyme levels in the presence of *LAC9* was not as severe as in the presence of *GAL4*. We conclude that *LAC9* is able to activate transcription of the melibiose-galactose regulon in *S. cerevisiae*. However, *LAC9* behaves uniquely and does not simply mimic *GAL4*.

DISCUSSION

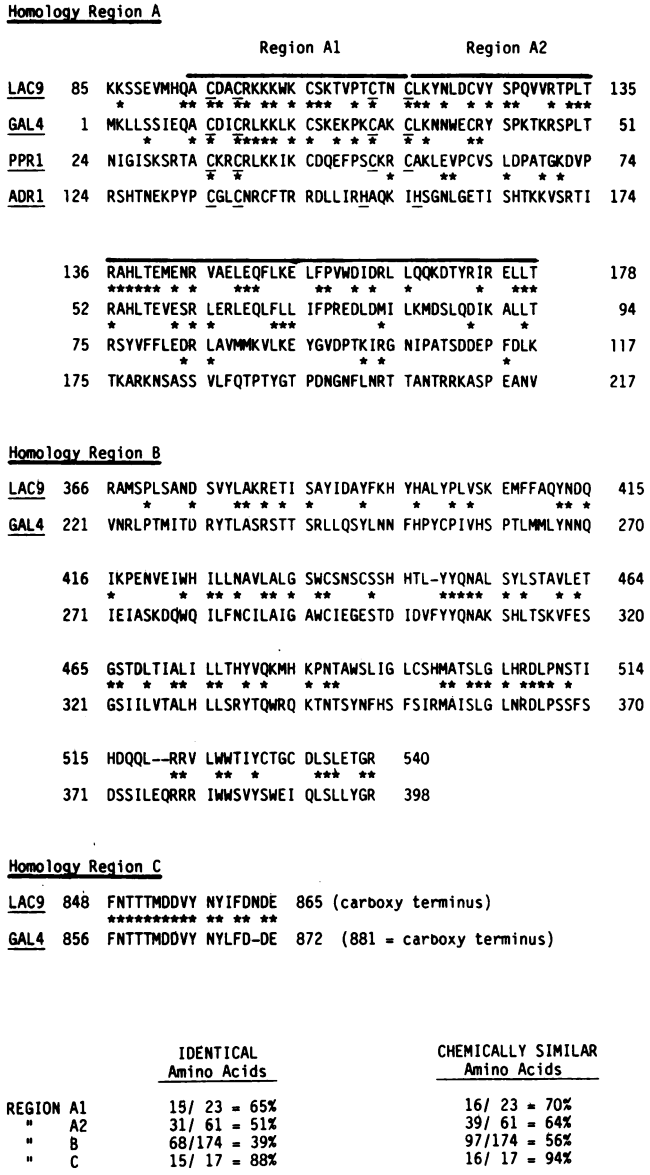
The evidence presented in this paper leads us to conclude that *LAC9* is a positive regulatory gene that controls expression of the lactose-galactose regulon of *K. lactis*. This conclusion is based on the phenotype of *lac9* mutants, which are unable to induce any of the lactose-inducible enzyme activities, and on the ability of *LAC9* to complement a *gal4* defective strain of *S. cerevisiae*. As we discuss below, the *LAC9* protein most likely works to activate transcription by binding to a UAS located in front of target genes.

The organizational and phenomenological similarities between the lactose-galactose regulon of *K. lactis* and the melibiose-galactose regulon of *S. cerevisiae* (37) suggested the existence of structural and functional similarities. As the data in this paper demonstrate, the LAC9 protein of *K. lactis* and the GAL4 protein of *S. cerevisiae* show both functional and structural similarities. The function of these proteins was compared by examining the ability of *LAC9* to complement a *gal4* strain of *S. cerevisiae*. *LAC9* activated expression of *GAL1*, *GAL2*, *GAL7*, and *GAL10*, since the *gal4* defective strain carrying *LAC9* on a *CEN* vector (YCp50) grew on galactose as well as the same strain carrying *GAL4* on YCp50. *LAC9* was also able to activate expression of *MEL1* as well as *GAL4* (Table 5). We conclude that *LAC9* and *GAL4* activate transcription by a common mechanism which we discuss in more detail below.

The data shown in Table 5 indicate that *LAC9* may not simply mimic *GAL4* but rather that it may confer unique phenotypes on *S. cerevisiae*. First, *MEL1* and *GAL1* were constitutively expressed in the presence of *LAC9* but not in the presence of *GAL4*. Second, glucose repression of *MEL1* and, in particular, *GAL1* was less severe in the presence of *LAC9* than in the presence of *GAL4*. Both of these observations could be explained by a high concentration of LAC9 protein, but the concentration would have to be extremely high compared with GAL4 to account for such a high level of constitutivity (21). Another explanation for these results would argue that they are due to mixing of mutant GAL4 subunits with normal LAC9 subunits to form a heteromeric regulatory protein. The *gal4* mutation in strain SJ21 is most likely a point mutation (21), so the strain probably makes mutant GAL4 protein.

An alternative explanation for the constitutive enzyme levels and reduced glucose repression is that the LAC9 protein does not interact with components of the melibiose-galactose regulon in the same manner as does the GAL4 protein. For example, the constitutive phenotype may indicate that the LAC9 protein does not interact with the negative regulatory protein GAL80. The GAL80 protein is thought to prevent constitutive expression of the melibiose-galactose regulon by interacting with GAL4 protein (21, 47).

The reduced glucose repression of *GAL1* and *MEL1* expression seen in the presence of *LAC9* may indicate that the LAC9 protein does not respond as well to the glucose repression circuit of *S. cerevisiae* as does the GAL4 protein. A direct implication of the data in Table 5 is that GAL4



	IDENTICAL Amino Acids	CHEMICALLY SIMILAR Amino Acids
REGION A1	15/ 23 = 65%	16/ 23 = 70%
" A2	31/ 61 = 51%	39/ 61 = 64%
" B	68/174 = 39%	97/174 = 56%
" C	15/ 17 = 88%	16/ 17 = 94%

FIG. 4. Amino acid sequence homologies between the LAC9 and GAL4 proteins. The amino acid sequences of the LAC9 and GAL4 proteins were compared by the procedures of Wilbur and Lipman (49) as modified by BIONET. Amino acid residues that are identical in the LAC9 or GAL4 proteins are indicated by an asterisk. Amino acid residues in the PPR1 or ADR1 proteins that are identical to either LAC9 or GAL4 protein are indicated by an asterisk also. The underlined C and H residues are those in the proposed metal-binding finger for the GAL4, PPR1, and ADR1 proteins (18). A minimum number of gaps have been introduced to produce the homologies shown. Slightly higher percent homologies could be obtained by introducing a larger number of gaps. Chemically similar amino acids were grouped as follows: Ala, Gly, Pro, Ser, Thr; Asp, Glu, Asn, Gln; Phe, Trp, Tyr; His, Lys, Arg; Ile, Leu, Met, Val.

protein plays a role in glucose repression. From circumstantial evidence, such a role has been proposed previously by Matsumoto et al. (33). They suggested that glucose represses gene expression via three pathways, one of which uses *GAL4*. This model predicts that full glucose repression requires all three pathways. Inactivation of one pathway should only partially relieve glucose repression, and this is exactly what we observed with *LAC9* in a *gal4* strain (Table 5).

Our recent data suggest a role for *GAL4* in glucose repression. When *GAL4* was introduced into a *lac9* defective strain of *K. lactis*, a greater than 30-fold level of glucose repression was observed for the lactose-galactose regulon (38). In contrast, a normal *LAC9* strain showed only slight, twofold, glucose repression of the regulon.

Comparison of the predicted LAC9 protein sequence with the GAL4 protein sequence revealed three regions of homology which account for about 30% of the amino acids in the proteins (Fig. 4). One of these regions involves the amino terminus of the proteins (Fig. 4, region A). It is known that the DNA-binding domain of GAL4 protein lies within the first 74 amino-terminal amino acid residues (17). This homology combined with the gene activation data (Table 5) imply that the GAL4 and LAC9 proteins have similar DNA-binding domains and should recognize similar UASs. Giniger et al. (17) identified a family of UASs and defined a consensus sequence that regulates transcription of the melibiose-galactose regulon of *S. cerevisiae*. We searched for and found a related UAS in the promoter of the β-galactosidase gene, *LAC4*, of *K. lactis*. The UAS is probably functional since it falls in a region of the promoter that is necessary for induction of β-galactosidase activity (9; S. Bhairi, Ph.D. thesis, University of Kentucky, Lexington, 1984). The sequences are:

S. cerevisiae C G G A A G A C T C T C C T C C G (consensus)
K. lactis C G G A A A T T T G T G G T C C G (*LAC4*)

The most conserved bases (100%) in the *S. cerevisiae* UAS family are the terminal CGG and CCG (5). These same bases are present in the *K. lactis* UAS, and they probably form important contacts with the GAL4 and LAC9 proteins. Other less conserved bases may form contacts with the proteins as well.

We noted in Fig. 3 that a UAS-type sequence is located about 400 base pairs in front of *LAC9* and suggested that transcription of *LAC9* might be regulated by binding of the LAC9 protein to this UAS. Alternatively, this UAS could regulate transcription of a gene located upstream of *LAC9* and oriented in the opposite direction. Since *LAC10* is linked to *LAC9*, the UAS could regulate *LAC10* expression.

An interesting feature of the amino-terminal homology region (Fig. 4, region A) is that it includes not only the proposed metal-binding finger (18), but an adjacent region (Fig. 4, region A2). To try and understand the significance of this homology we compared this region of the GAL4 and the LAC9 proteins with a corresponding region in two other yeast positive regulatory proteins, ADR1 (18) and PPR1 (23). Hartshorne et al. (18) have shown that the GAL4, ADR1, and PPR1 proteins have a similar metal-binding domain (region A1 in Fig. 4). Our comparison of the adjacent region, region A2, indicates that LAC9 protein is more homologous to GAL4 protein (31 of 63 amino acids are identical) than to either the PPR1 protein (11 of 63 amino acids are identical to LAC9 protein) or the ADR1 protein (6 of 63 amino acids are identical to LAC9 protein). On the basis of this comparison and the fact that the GAL4, ADR1, and PPR1 proteins all recognize unique UASs, we hypothesize that while region A1, the metal-binding finger, is necessary for DNA binding, it is region A2 of the GAL4 and the LAC9 proteins that specifies binding to UASs.

TABLE 6. Codon usage in *LAC9*[a] and *GAL4*[b]

Codon	*LAC9*	*GAL4*	Codon	*LAC9*	*GAL4*
TTT-Phe	14	24	TCT-Ser	21	28
TTC-Phe	14	14	TCC-Ser	14	15
TTA-Leu	23	17	TCA-Ser	36	20
TTG-Leu	24	22	TCG-Ser	9	10
CTT-Leu	17	12	CCT-Pro	12	13
CTC-Leu	8	8	CCC-Pro	3	9
CTA-Leu	17	17	CCA-Pro	21	13
CTG-Leu	7	19	CCG-Pro	9	11
ATT-Ile	8	25	ACT-Thr	21	20
ATC-Ile	13	18	ACC-Thr	12	13
ATA-Ile	23	13	ACA-Thr	23	24
ATG-Met	18	19	ACG-Thr	11	9
GTT-Val	15	10	GCT-Ala	13	10
GTC-Val	4	10	GCC-Ala	12	12
GTA-Val	10	10	GCA-Ala	19	15
GTG-Val	6	9	GCG-Ala	12	6
TAT-Tyr	18	18	TGT-Cys	8	10
TAC-Tyr	9	9	TGC-Cys	8	8
TAA-	1	1	TGA-	0	0
TAG-	0	0	TGG-Trp	18	12
CAT-His	13	13	CGT-Arg	3	4
CAC-His	8	4	CGC-Arg	1	3
CAA-Gln	29	28	CGA-Arg	1	7
CAG-Gln	17	18	CGG-Arg	7	0
AAT-Asn	34	37	AGT-Ser	15	16
AAC-Asn	37	23	AGC-Ser	8	11
AAA-Lys	20	33	AGA-Arg	8	14
AAG-Lys	19	14	AGG-Arg	9	8
GAT-Asp	26	34	GGT-Gly	12	11
GAC-Asp	11	11	GGC-Gly	5	7
GAA-Glu	29	25	GGA-Gly	7	5
GAG-Glu	13	15	GGG-Gly	2	6

[a] 865 amino acids; molecular weight, 97,050.
[b] 881 amino acids; molecular weight, 99,350 (28).

The function of homology region B (Fig. 4) in the middle of the two proteins is not clear. It may mediate interaction with the GAL80 protein since *GAL4*[c] mutations map in or near the middle of *GAL4* (32). The corresponding region in LAC9 protein might interact with the negative regulator, LAC10 (14). Since region B is large it may have more than one function, but this remains to be determined. Other known functions that could be carried out by this region or region C include glucose repression (33), subunit interaction, and transcriptional activation (17). Also, some region of GAL4 protein may interact with the inducer (21 and references therein), and the same might hold for LAC9 protein.

The relatedness of the LAC9 and GAL4 proteins is also revealed by their similar amino acid composition and their codon usage (Table 6). Neither gene shows the codon bias observed for *S. cerevisiae* genes that are expressed at high levels (2, 42).

From an evolutionary point of view it seems reasonable to propose that the LAC9 and GAL4 proteins arose from a common ancestor. The three regions of homology probably perform functions that are under similar selective pressure in *K. lactis* and *S. cerevisiae*. More data are needed to determine whether the nonhomologous regions represent specialized domains that have been selected for or whether they are neutral regions that have little selective value to the host. Selection for specialized domains may have occurred in the two proteins since their host organisms have different habitats. *K. lactis* is found naturally in dairy products in which it can grow on lactose (48), a carbon source not used by *S. cerevisiae*. Also, as mentioned previously, glucose represses the lactose-galactose regulon of *K. lactis* only about twofold (12, 38) but does, however, severely repress other genes in the presence of glucose (29). In contrast, glucose severely represses the melibiose-galactose regulon of *S. cerevisiae* (1).

Further experiments aimed at exploiting the differences between *K. lactis* and *S. cerevisiae* should provide insight into the molecular mechanisms controlling the expression of the lactose-galactose and melibiose-galactose regulons.

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ADDENDUM

Salmeron and Johnston (Nucleic Acids Res. **14**:7767–7781, 1986) determined the nucleotide sequence of *LAC9* and examined the behavior of the gene in a *gal4* defective strain of *S. cerevisiae*. While their structural analysis is in agreement with ours, their data for the behavior of *LAC9* in *S. cerevisiae* are not in agreement with ours (Table 5). They observed low expression of *MEL1* (α-galactosidase activity) in the absence of inducer even when *LAC9* was carried on a multicopy vector. We observed high expression of *MEL1* when *LAC9* was present at one copy per cell. They observed induction of *MEL1* expression; we did not. They found that *MEL1* expression was as severely repressed by glucose in the presence of *LAC9* as in the presence of *GAL4*. We found a difference in the behavior of these two genes: less severe glucose repression was seen in the presence of *LAC9*. We think that all these differences are due to the *LAC9* DNA fragment used by Salmeron and Johnston. Their DNA fragment was defined at the 5′ end of *LAC9* by a *Bgl*II restriction site (nucleotide 905 in Fig. 3). Our data show that normal transcription of *LAC9* starts upstream of this site. Thus, the *LAC9* gene used by Salmeron and Johnston lacked a promoter.

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